(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number : **0 550 376 A1**

(12) EUROPEAN PATENT APPLICATION

(21) Application number : **92500145.5**

(51) Int. Cl.[5] : **A61K 37/54**, C12N 9/64

(22) Date of filing : **10.11.92**

(30) Priority : **08.11.91 ES 9102474**

(43) Date of publication of application :
07.07.93 Bulletin 93/27

(84) Designated Contracting States :
AT BE CH DE DK FR GB GR IE IT LI LU MC NL PT SE

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(54) Procedure for producing a polypeptide-type product having an inhibiting effect on hyperproduction of the tumor necrosis factor.

(57) The present invention consists of a procedure for producing a polypeptide-type product having an inhibiting effect on hyperproduction of the tumor necrosis factor, characterized in that a commercial pepsin preparation is subjected to a series of dialysis procedures, each of which is performed under defined conditions of time and temperature and with specified volumes and types of dialysis fluid, after which said product is dried by means of lyophilization, thereby yielding a product in the form of an hygroscopic white powder which lacks the original proteolytic activity, is not toxic, and which when administered orally inhibits the serum levels of TNF induced by liposaccharide (LPS), and which therefore is suitable for the manufacture of pharmaceutical preparations for oral use in human beings. Said procedure is applicable to the pharmaceuticals industry.

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The present invention relates to a procedure for producing a polypeptide-type molecule having an inhibiting effect on hyperproduction of the tumor necrosis factor (TNF) and which therefore is useful in the manufacture of pharmaceutical preparations. The advancement of scientific knowledge has made it possible to establish the participation of TNF in many pathological processes. TNF is normally produced by monocytes and/or macrophages. This cytokin, which was originally characterized by its tumor necrotic effect and which was subsequently identified as being homologous with cachectin, today is included in the group of substances known as inflammatory cytokins, i.e., those which are produced at inflammation sites by infiltrating mononuclear cells (Jan Vilcek and Tae H. Lee, in J. Biol. Chem., Vol. 266 (1991), at pages 7313 to 7316). Its participation in modulating the manifestation of polymorphonuclear leukocyte adhesion antigens in this context has been described. The hyperproduction of TNF- α has been described in a large number of pathologies, such as cachexia, septic shock, rheumatoid arthritis due to other autoimmune disorders, parasitic infections, viral infections, etc. Therefore it is important that products be available which are pharmacologically equipped with the ability to inhibit the hyperproduction of TNF. In this context, the inhibition of the production of TNF has been described in various experimental models. For instance, E. Sampaio et al. (in J. Exp. Med., Vol. 173 (1991), at pages 699 to 703) describe the inhibition by means of thalidomide, of the production of TNF by human monocytes stimulated in vitro by lipopolysaccharide (LPS); M. Gardina et al. (in J. Exp. Med., Vol. 173 (1991), at pages 1305 to 1310) describe the effect of chlorpromazine, which consists of the reduction of serum levels of TNF in animals stimulated by LPS; S. Billy et al. (in Int. J. Immunopharm., Vol. 12 (1991), at pages 31 to 36) have described the inhibiting effect of quinolones on the production of TNF by human monocytes. Similar effects have been described for indomethacin. The literature contains descriptions of the pharmacological properties of pepsin administered intravenously and with regard to its characteristic effect, i.e., the proteolytic effect. These include the effect on the formation of dental plaque (H.G. Scheider, E. Goebbels, and K. Puechner, in Stomatol. DDR, Vol. 36 (1986), at pages 433 to 438), on Masussi's nephritis (H. Ohnishi, in Nippon Yakurigaku Zasshi, Vol. 83 (1984), at pages 105 to 114), on autoimmune disorders in murine models (H. Ohnishi, in Life Sci., Vol. 33 (1983), at pages 1641 to 1648), and on glomerulonephritis as produced by immunocoplexes (H. Ohishi SIC , in Life Sci., Vol. 33 (1983), at pages 671 to 677).

The present invention describes a procedure for producing, from pepsin, a polypeptide-type product which has no proteolytic effect and which, when administered in the proper manner orally to mice, is capable of partially inhibiting the serum levels of TNF induced by bacterial endotoxin (LPS).

GENERAL DESCRIPTION OF THE PROCEDURE

The procedure for the production of a polypeptide-type product having an inhibiting effect on hyperproduction of the tumor necrosis factor (TNF), which is the object of the present invention, consists of a process of exhaustive and successive dialyses of an aqueous solution of a pepsin preparation, said aqueous solution having a defined richness, and with said procedure ocurring in three stages for which the time, the type and volumes of dialysis liquid, and the temperature are defined, followed by a process of drying by means of lyophilization.

During the process performed under the controlled conditions as described herein, the initial protein is modified by means of autolysis and denaturation with the loss of the characteristic initial proteolytic effect, and the new clinically applicable pharmacological property is obtained. The stages of said procedure are described below.

- In the first stage, a solution of said pepsin preparation is prepared in deionized water, with said solution having a final richness of 50 to 200 mg per ml^{-1}, with said solution being filtered if necessary.
- In the second stage, after the solution obtained in the preceding stage has been introduced into a Visking type dialysis container having a molecular length of 6 to 14 kd, the first dialysis is performed with running water over a period of time of 2 to 6 days and at room temperature.
- In the third stage, the second dialysis is performed, with a volume of deionized water which is from 50 to 1000 times the volume of the liquid to be dialyzed, over a period of time of 4 to 20 hours and at temperature of 4 to 10ºC.
- In the fourth stage, the third dialysis is performed, with a volume of distilled water which is from 50 to 1000 times the volume of the liquid to be dialyzed, over a period of time of 8 to 30 hours and at temperature of 4 to 10ºC.
- In the fifth stage, the dialisys liquid is frozen and dried by means of lyophilization.
- In the sixth stage, the dried product obtained in the previous stage is used to prepare galenic forms of the product which are appropriate for oral or intravenous administration, with said forms being the ones which will be used in various biological tests. For oral administration, solid mixtures containing 1 to 5 percent $CaH(PO_4) \cdot 2H_2O$ are prepared. For intravenous (IV) administration, extemporaneous solutions

are prepared which have a richness of 2 to 5 μg per ml^{-1} in an isotonic saline solution.

EXAMPLE

In a first stage, a quantity of 0.1 to 2.5 grams of commercial pepsin at a grade of 1:3000 is weighed and dissolved in a volume of 10 to 200 ml of deionized water. If the resulting solution is not clear, it is filtered through a 0.45 μ cellulose filter.

In a second stage, said aqueous pepsin solution is introduced into a Visking type dialysis container having a molecular length in the range from 10 to 14 kd and which was first washed in water at a temperature of 60ºC and then exhaustively rinsed with distilled water. A flask is filled with running water and introduced into said dialysis container, and the mouth of said flask is placed under a stream of running water having a slow flow rate (e.g., 20 to 40 ml per $minute^{-1}$), thus ensuring the continuous renewal of said dialysis fluid. Said step is performed at room temperature and lasts for a period of 2 to 6 days.

In a third stage, said dialysis container containing said dialyzed pepsin solution from the preceding stage is dialyzed with a quantity of 1 to 10 liters of distilled water over a period of 4 to 20 hours and at a temperature of 4 to 10ºC.

In the fourth stage, said dialysis container containing said dialyzed pepsin solution from the preceding stage is dialyzed again, this time with a quantity of 1 to 10 liters of distilled water over a period of 8 to 30 hours and at a temperature of 4 to 10ºC.

In the fifth stage, the dialyzed pepsin solution obtained in the preceding stage is frozen and lyophilized, and an hygroscopic white powder is obtained.

In the sixth stage, the galenic forms of the product for pharmacological tests are prepared. For oral administration, a quantity of 200 mg of the lyophilized product is weighed and mixed with a quantity of 10 grams of $CaH(PO_4) \cdot 2H_2O$. Said mixing is achieved by means of mechanical stirring. For IV administration, a solution is prepared which has a richness of 2 to 5 μg per ml^{-1} in an isotonic saline fluid.

The product obtained after said fifth stage, and more particulary the product described in the preceding example, has new biochemical and pharmacological characteristics which are different from those of the initial product. Said characteristics are shown in Table I below.

TABLE I - BIOCHEMICAL PROPERTIES

Product	ϵ 1% 1 cm, 280 nm	Flourescence intensity at 340 nm (in water) (exc. 270 nm)	Percentage of protein (by weight) (as determined in accordance with Lowry*)	Percentage of sugars (by weight) (as determined in accordance with Dubois**)	Proteolytic activity (in arbitrary) (units) (as determined in accordance with Rinderknecht***)
Initial	1.3	100	19	68	100
Final	6.8	500 to 600	95	5	0 to 20

* O.H. Lowry, et al., in J. Biol. Chem., Vol 193 (1951), at pages 265 to 275.

** A. Dubois, K.A. gilles, et al., in Anal. Chem., Vol. 28 (1956), at pages 350 to 356.

*** H. Rinderknecht, et al., in Clin. Chim. Acta Vol. 21 (1968), at pages 197 to 203.

The product obtained after said sixth stage of the procedure has been carried out, and which is the subject of the present invention, has the following biological effects:

1) The product, in the form of a 2 percent mixture with phosphate salt and administered at a dosage of 150 mg per kg^{-1} to Balb/c mice over a period of 6 consecutive days, is capable of inhibiting 75 percent of the serum levels of TNF as induced by the IV injection of LPS administered 2.5 hours after administration of the last dose of the product.

2) The product, in the form of a solid 2 percent mixture with phosphate salt and administered at a dosage of 150 mg per kg^{-1} to Balb/c mice over a period of 1 or 4 consecutive days, produces an increase in the plasma level of corticosterone, said increase being estimated at 2 to 3 times the basal level 5 hours after the last dose.

3) The product, in isotonic saline solution, was administered to Swiss mice at a single dose IV of 0.05 mg per kg^{-1} and did not produce any anomalies in body weight or in the histology of the liver, spleen, kidneys, or mesenteric lymph nodes, either at 7 days or at 14 days after administration.

Claims

1. Procedure for producing a polypeptide-type product having an inhibiting effect on hyperproduction of the tumor necrosis factor (TNF), characterized in that:
   a) An aqueous pepsin solution is prepared which has a defined richness.
   b) Said solution is dialyzed (in a first dialysis) with running water under defined conditions of time and

temperature, and with a specified type of dialysis membrane.
c) The solution dialyzed in the preceding step is dialyzed again (in a second dialysis) with deionized water under defined conditions of time and temperature, and with a specified volume of dialysis fluid.
d) Dialisys is performed again (in a third dialysis) with distilled water under defined conditions of time and temperature, and with specified volume of dialysis fluid.
e) The dialysate obtained in the preceding step is dried.
f) Galenic forms are prepared which are appropriate for oral administration after dilution in the solid phase with calcium salts, or which are appropriate for intravenous administration by means of dilution in an isotonic saline solution.

2. Procedure for producing a polypeptide-type product having an inhibiting effect on hyperproduction of the tumor necrosis factor (TNF) in accordance with Claim 1, characterized in that the pepsin which is used is from type 1:2500 to type 1:60,000, and preferably type 1:3000, and by the fact that the aqueous solution which will be used in said dialysis process has a richness in said pepsin in the range from 10 to 125 mg per ml^{-1}.

3. Procedure for producing a polypeptide-type product having an inhibiting effect on hyperproduction of the tumor necrosis factor (TNF) in accordance with Claim 1, characterized by the fact that the conditions for said first dialysis are the following: said dialysis fluid is running water at a flow rate of 20 to 40 ml^{-1} per minute; said time is from 4 to 20 hours; and said temperature is room temperature.

4. Procedure for producing a polypeptide-type product having an inhibiting effect on hyperproduction of the tumor necrosis factor (TNF) in accordance with Claim 1, characterized in that the conditions for said second dialysis are the following: said dialysis fluid is distilled water; said volume of said dialysis fluid is from 50 to 1000 times the volume of the fluid to be dialyzed; said dialysis time is from 4 to 20 hours; and said temperature during said process is from 4 to 10ºC.

5. Procedure for producing a polypeptide-type product having an inhibiting effect on hyperproduction of the tumor necrosis factor (TNF in accordance with Claim 1, characterized in that the conditions for said third dialysis are the following: said dialysis fluid is distilled water; said volume of said dialysis fluid is from 50 to 1000 times the volume of the fluid to be dialyzed; said dialysis time is from 8 to 30 hours; and said temperature during said process is from 4 to 10ºC.

6. Procedure for producing a polypeptide-type product having an inhibiting effect on hyperproduction of the tumor necrosis factor (TNF) in accordance with Claim 1, characterized in that the dialysate obtained at the end of the process described in the preceding claim is dried by means of lyophilization and is pharmacologically active when administered orally in the form of a solid mixture with calcium salts.

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EUROPEAN SEARCH REPORT

Application Number
EP 92 50 0145

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	EP-A-0 100 366 (MOCHIDA PHARMACEUTICAL CO) 15 February 1984 ---	1-6	A61K37/54 C12N9/64
A	US-A-4 591 504 (H. OHNISHI ET AL) 27 May 1986 -----	1-6	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

C12N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	21 APRIL 1993	VAN DER SCHAAL C.A.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
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L : document cited for other reasons

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EPO FORM 1503 03.82 (P0401)